STUDIES ON THE ACROSOME. I. REACTION TO EGG-WATER AND OTHER STIMULI [1]

J. C. DAN

Tokyo Metropolitan University, Meguro-ku, Tokyo, and Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan

Our knowledge of spermatozoa and their role in the fertilization reaction has been acquired through studies approaching the subject from two widely different angles—"straight" morphological observation dealing with fixed specimens, such as the classical drawings of Retzius, or the electron microscope photographs of Bretschneider; and physiological studies centering around the fertilizin theory of F. R. Lillie and currently being pushed close to conclusion by the penetrating biochemical research of Tyler, Hartmann, Runnström and their co-workers.

One previous attempt to correlate the morphological and physiological aspects of sperm agglutination has been presented in the paper of Rötheli, Roth and Medem (1950), which deals with several species of freshwater fishes. The reaction in these forms, however, is an irreversible one, in which the spermatozoa become more and more firmly aggregated into increasingly dense clumps, and it seems difficult to distinguish possible normal changes in form due to agglutination from those accompanying the death of the spermatozoa.

In sea urchins, because of the small size and violent activity of spermatozoa in their normal state, work on living sperm has almost necessarily dealt with them in bulk suspensions; the vital staining study by Popa in 1927 is a rare example of an effort to examine living spermatozoa one at a time. A tendency in this direction is also to be found in Carter's 1931 paper, in which he stresses the fact that considerable physiological differences can be discerned between the gametes of a single induced shedding.

With the development of the phase contrast microscope, it has become possible to distinguish hitherto invisible details of active spermatozoa in a sufficiently normal state that observations can be made of their behavior under various conditions (Dan, 1950). Phase microscopy also provides a stepping-stone to the use of the electron microscope, by permitting a large degree of comparison between the living state and the electron photographs, and to a great extent obviating the uncertainty concerning the appearance of artifacts which would otherwise inevitably attach to a method involving observation *in vacuo*. All the points discussed in this paper have been checked in the living condition and in spermatozoa immediately after fixation in sea water suspension, and it can be stated with confidence that no qualitative difference was found among the three types of observation.

Materials and Methods

The spermatozoa photographed in this study belong to the two sea urchin species, *Pseudocentrotus depressus* and *Strongylocentrotus pulcherrimus*, although

[1] This research was supported in part by the Ministry of Education Research Expenditure.

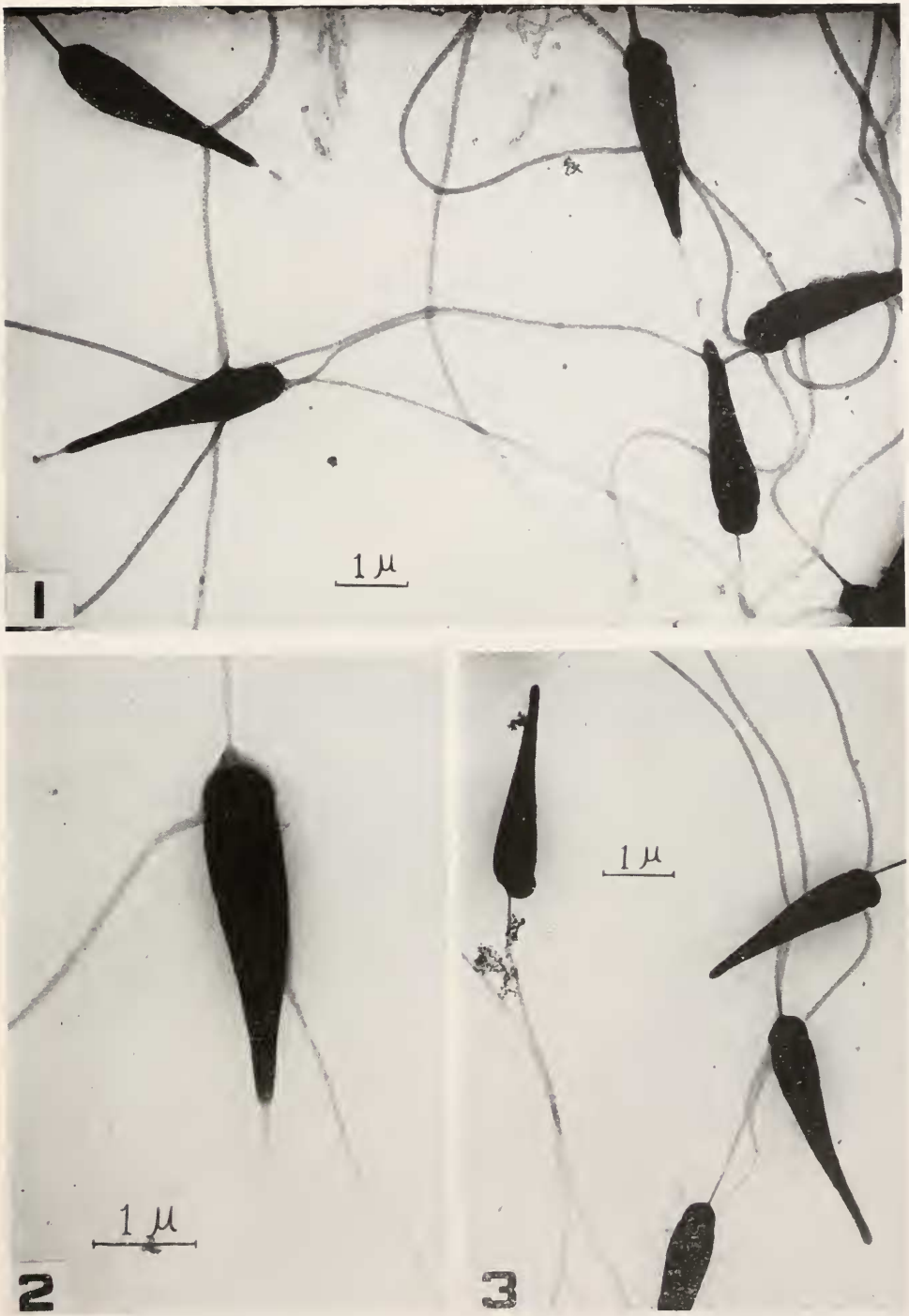

FIGURE 1. Spermatozoa of *Pseudocentrotus depressus*, fixed 30 seconds after the addition of egg-water.

FIGURE 2. Same as Figure 1.

FIGURE 3. Control in ordinary sea water.

phase contrast observation has shown that the phenomenon in question is generally characteristic of the other echinoids available at Misaki.

In every case, suspensions were made from dry sperm secured by removal of the testes with care to avoid contamination by sea water or body fluid; only freshly suspended spermatozoa were used. Egg-water was obtained by inducing shedding of eggs into filtered sea water (by electrical stimulation of intact females in the case of Pseudocentrotus (see Iwata, 1950), and by introducing a few drops of isotonic KCl into the cut test of Strongylocentrotus); the suspension was stirred occasionally during a minimum of two hours, and the titer of the supernatant jelly-sea water solution roughly determined by low-power microscopic observation of the intensity and duration of the agglutination reaction which occurred when a given amount of this egg-water was mixed with the standard sperm suspension (ca. 0.025 cc. dry sperm in 2 cc. sea water).

Irreversible aggregation of the spermatozoa was induced by suspending them directly in sea water of pH 9.2. For the first 30 seconds no heterogeneity could be detected in the distribution; from 40 seconds a tendency to clumping was apparent; by two minutes most of the spermatozoa were involved in aggregations approximately the size of those formed at the height of the reversible agglutination reaction. The suspension was fixed at this stage.

The spermatozoa were fixed by the addition of neutralized formalin to the suspensions; they were left for at least 24 hours in this solution before dilution of the sea water. The change to 5–7% formalin in distilled water was carried out by allowing the suspended spermatozoa to settle naturally, since it was found that even gentle centrifuging caused clumping.

Electron photographs were taken with a Hitachi standard type electron microscope, at an operating voltage of 50 KV. Shadowing was done with Cr_2O_3.

Results

In both species, the agglutination reaction occurs instantanously upon addition of egg-water to a sperm suspension; at the titer chosen as standard, the clumping of spermatozoa is at its maximum—*i.e.*, the greatest numbers of spermatozoa are involved in the clumps—at about 30 seconds after addition of the egg-water, and dispersal is complete within 2½–4 minutes. Figures 1 and 2 show spermatozoa fixed at the height of the agglutination reaction; Figure 3 is a photograph of spermatozoa similiarly suspended and fixed 30 seconds after the addition of the same amount of plain sea water. It is clear that a change has occurred in the region of the acrosome, under the influence of the egg-water; the probable nature of this change will be discussed below.

In Figures 4 and 5 are shown two photographs of Pseudocentrotus spermatozoa fixed 5 seconds after the addition of egg-water. It is important that in spermatozoa thus fixed immediately after reaction to the egg-water stimulus, the acrosome sub-

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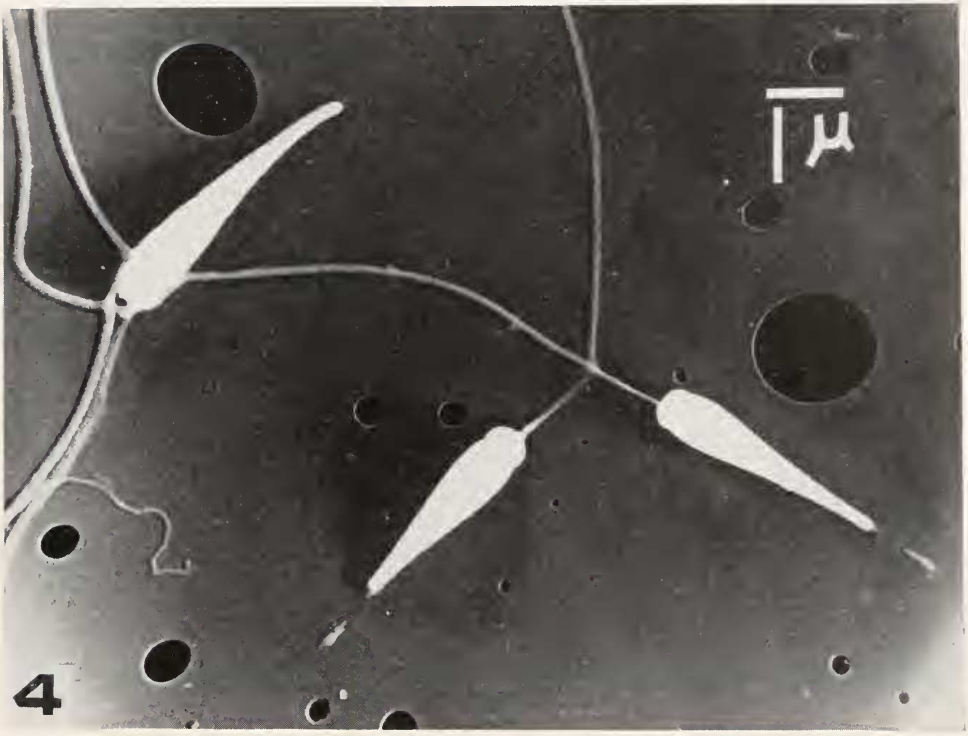
Figure 4. Pseudocentrotus spermatozoa fixed 5 seconds after addition of egg-water. The spermatozoan at the upper left has failed to react. The breakage between the head of the spermatozoan and the extruded acrosome substance is caused by drying on the collodion membrane. Shadowed with Cr_2O_3, $\tan^{-1} = \frac{1}{2}$.

Figure 5. Same as Figure 4, magnification 32,000 ×.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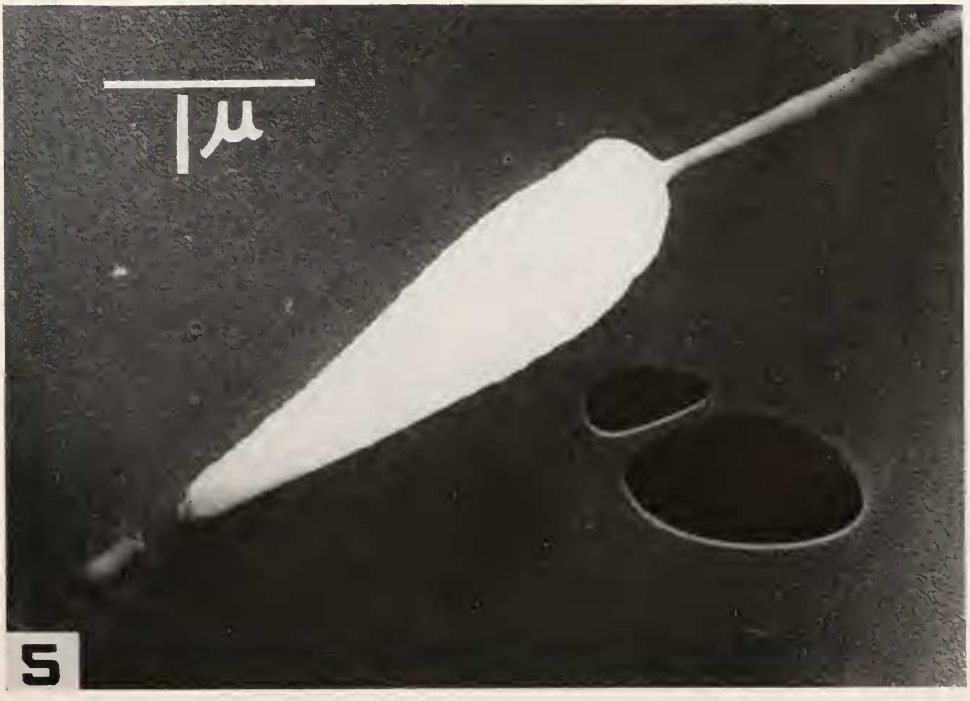
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stance appears as a drop-like mass on the tip of the head. The break which almost invariably occurs between the tip of the sperm and the substance attached to it in such early fixation is interpreted as an artifact due to the contraction of the preparation on drying. Comparison of these photographs with Figures 6 and 7, of spermatozoa fixed at 2 minutes 35 seconds after reversal of agglutination, gives the impression that part of the acrosome substance disperses to a considerable extent, while a central core or fiber of some sort (which may be a separate structure that appears secondarily), possessing greater cohesiveness and not dispersing readily in sea water, remains attached to the former acrosomal region.

Figures 8 and 9 show photographs of Strongylocentrotus spermatozoa, fixed 25 seconds after addition of egg-water and plain sea water, respectively, to fresh suspensions. Figure 10, of spermatozoa fixed after two minutes in sea water at pH 9.2 indicates that morphologically the same sort of reaction is called forth by alkalinity as by egg-water.

Figures 11 through 15 are photographs of spermatozoa which were placed in living suspension (in plain sea water) directly onto a collodion membrane mounted for electron microscope observation, in order to test the possibility that the reaction of the acrosome may be induced by simple contact with solid objects, in the absence of egg jelly substance. The sperm were observed with low power of an ordinary microscope to ascertain that most of them were attached to the membrane by their tips and rotating. A drop of formalin-sea water was then added, at 105 seconds after suspension, and changed several times to remove unattached spermatozoa; after three hours fixation in formalin-sea water, the preparation was washed with dilute formalin and then distilled water before desiccation.

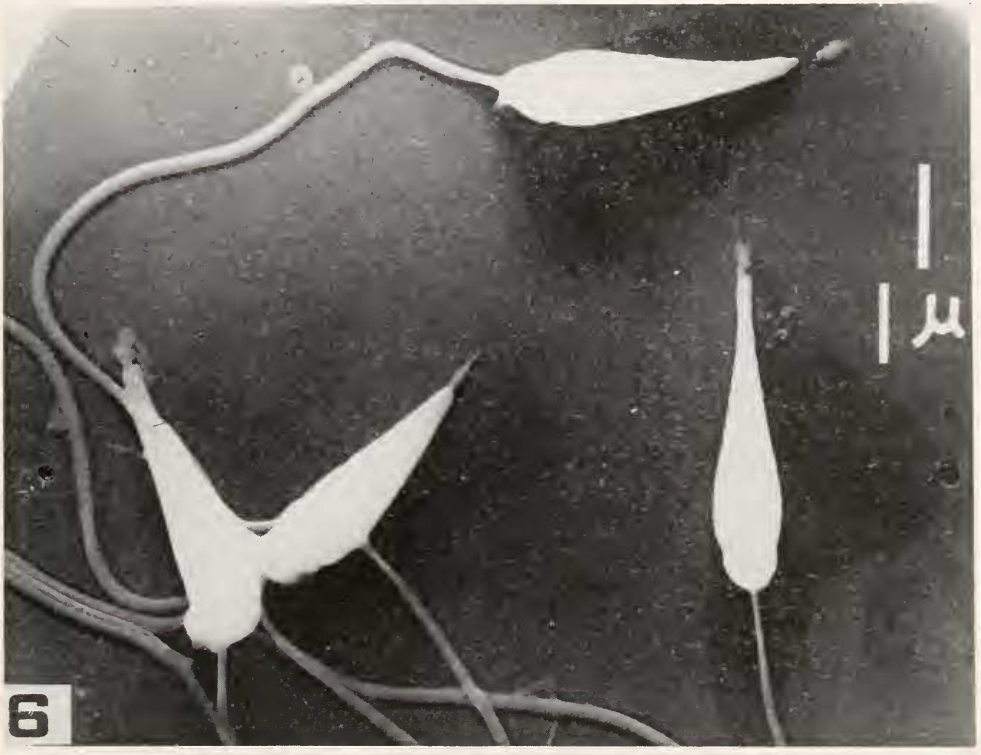
The photographs show that attachment to the collodion membrane induces a reaction essentially the same as that produced by egg-water. The acrosome substance is set free, appearing as an amorphous mass which fails to form a clear boundary in sea water and tends to spread on the collodion surface.

Discussion

Popa, after staining living *Arbacia punctulata* spermatozoa with Janus green, reports (p. 251), ". . . one gets the impression that the spermatozoa eliminates through the point of the head (where really there is an exceedingly minute opening) very small amounts of an extremely sticky substance." In egg-water (p. 256), "This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule."

With phase contrast oil immersion, it is easy to recognize Popa's "small granule" at the tip of the acrosome of unstained spermatozoa freshly suspended in sea water, of several sea urchin species. It is also just barely possible to make out a small mass of colorless, non-refringent substance attached to the tips of spermatozoa in egg-water (after reversal of agglutination, or immediately after addition of the egg-water if this is done under a cover-glass so that the spermatozoa become attached

Figures 6 and 7. Pseudocentrotus sperm fixed after reversal of agglutination (2 minutes 35 seconds after addition of egg-water).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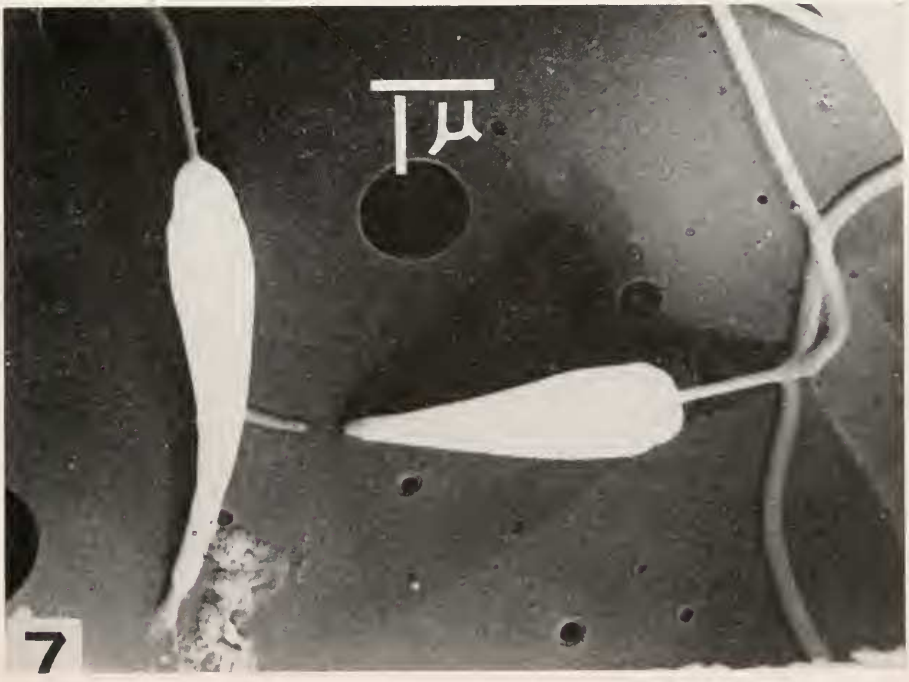
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to the glass surfaces instead of forming clumps). When such spermatozoa are freely suspended, the substance appears as a flabby, gelatinous "tongue" of uniform diameter (roughly 0.2 μ), which may reach a length of more than 1 μ and is always in vibratory, Brownian-like motion. That it is exceptionally sticky in normal sea water is attested to by the fact that once this substance has come into contact with any surface, it is never pulled loose even by the most vigorous movements of the spermatozoan. A few cases have been observed in which active spermatozoa have broken away, leaving at least part of the "tethering" substance on the glass surface, but it is much more common to find them permanently attached by the highly pliable strand which obviously possesses considerable cohesiveness.

As for Popa's "minute opening," however, it is probable that what he was seeing was rather a denser portion at the extreme tip of the acrosome, which appears darker than the more proximal part with phase contrast.

Before this study reached the stage of electron microscopy, it seemed fairly apparent that a gelatinous substance was extruded from the tip of the acrosome in response to the stimulus of egg-water, alkaline sea water and contact with solid surfaces. However, careful study of all the electron photographs seems to indicate rather that the anterior part of the acrosome undergoes a drastic change within one or two seconds after such stimulation. This is most clearly shown in Figure 4, in which the one spermatozoan which has failed to respond immediately to the egg-water stimulus is long and tapering at the apex, while the two others which show the typical reaction are truncated.

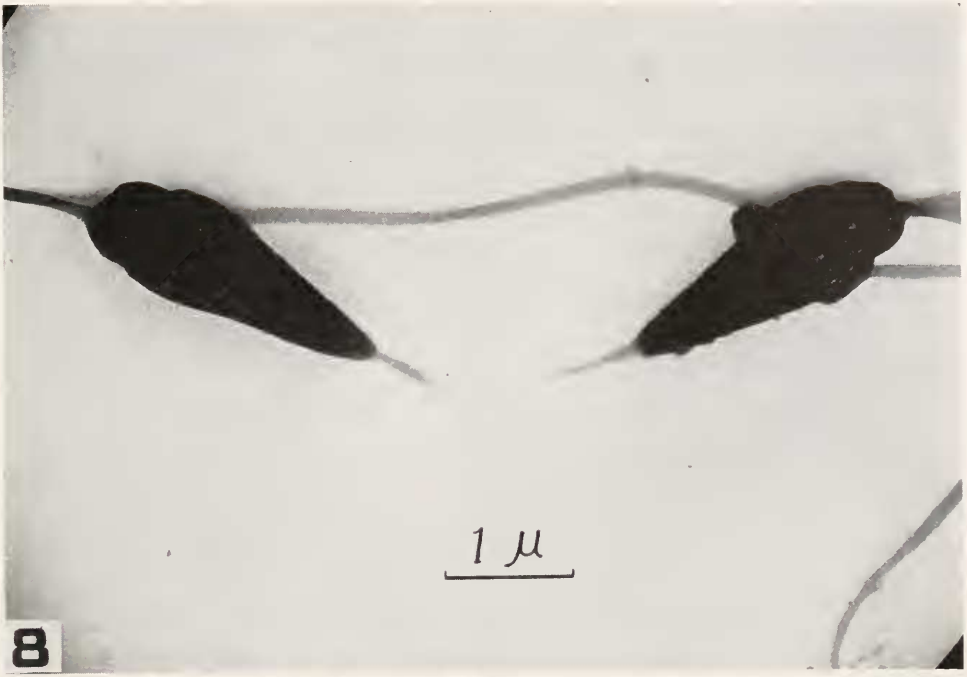
It is proposed that the condition of the acrosomal region in sperm which have so reacted (*cf.* Fig. 5) can best be explained by imagining that the plasma membrane investing the tip of the acrosome undergoes local autolysis, leaving the underlying substance exposed. If this substance is, or includes, an *egg-membrane* lysin such as that isolated by Tyler (1939) and Krauss (1950a, 1950b), or an *egg-surface* lysin like Androgamone III of Runnström *et al.* (1944, 1945, 1946), the postulated breakdown of the acrosome membrane would leave the lysin free to attack the vitelline membrane of the egg as soon as the spermatozoan carried it into contact with the egg surface.

The two widely differing lytic substances identified by these two groups of workers are obtained from frozen-thawed or frozen-dried sperm; there has so far been presented no unequivocal evidence as to their ready availability or exact location in the living spermatozoan, although Tyler (1949) has published electron micrographs of keyhole limpet spermatozoa showing that extraction with alkaline sea water (pH 9), which yields a solution of the egg membrane lysin, causes a more or less drastic breakdown of the acrosome. Pending tests of its chemical properties and lytic activity, it can only be offered as a surmise that the acrosome substance under discussion in this paper contains either or both of the lysins identified by Tyler and Runnström.

FIGURE 8. Strongylocentrotus spermatozoa fixed 30 seconds after agglutination with egg-water.

FIGURE 9. Strongylocentrotus sperm fixed 30 seconds after addition of plain sea water to suspension (control).

FIGURE 10. Strongylocentrotus sperm fixed after 2 minutes in sea water of pH 9.2.


1 μ
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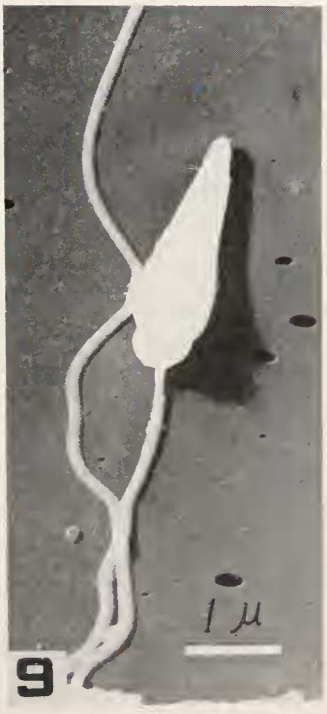
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10

In support of that surmise, certain circumstantial evidence can be adduced. The time relationships satisfactorily fit the requirements of the process as it must occur in normal fertilization, if it is assumed that the acrosome membrane is stimulated by the jelly substance, either intact or in solution in the immediate vicinity of the egg, and reacts during the very few seconds which are required for the spermatozoan to pass through the jelly layer and reach the vitelline membrane.

The fact that this reaction of the acrosome is also induced by hyperalkalinity in the absence of the species specific factor contained in the egg jelly would account for the long-known empirical fact that raising the pH of the medium facilitates sperm penetration in inter-species crosses, self-fertilization in self-sterile hermaphrodites, and other cases in which fertilization is blocked in normal sea water.

Furthermore, the observed reaction to contact with solid objects offers a fairly satisfactory explanation for the fact that removal of virtually all the jelly from unfertilized eggs by acid extraction does not prevent fertilization.

On the basis of these various observations, it is proposed that there are two separate phenomena simultaneously involved in egg-water agglutination of sea urchin sperm: a specific reaction (fertilizin-antifertilizin) which orients the spermatozoa into regular groups and holds them so for a definite period; and a response of the acrosome to the chemical stimulation of the dissolved jelly, which is manifested by an almost instantaneous local breakdown of the acrosome membrane so that the acrosome substance is exposed at the tip of the sperm head as a relatively labile mass. This response of the acrosome is believed to be less specific in nature than the agglutination reaction, being induced also by hyperalkalinity and (possibly somewhat less readily) by contact with solid surfaces.

Applying this experimental conclusion to natural fertilization, it is suggested that as the spermatozoan actively swims through the loose network of the jelly layer, it responds to the chemical stimulation of the jelly substance by a breakdown of the membrane covering the front part of the acrosome, so that by the time the sperm reaches the egg surface, a few seconds later, it carries at its tip a mass of freshly exposed lysin with which it effects penetration of the vitelline membrane as the first step in the fertilization process.

In a discussion of the properties of fertilizin, Tyler (1949) makes the following statement (p. 203): ". . . experiments show that fertilizin acts as an aid to fertilization, but only when it is present on the surface of the egg, as the gelatinous coat. . . . Evidently when the interaction of the egg and fertilizin takes place away from the egg and is completed before the sperm reaches the surface of the egg, the sperm are then unable to combine with the egg." Assuming that the sea water-labile portion of the acrosome substance is a vitelline membrane lysin, its demonstrated dispersal within less than 30 seconds after contact with egg-water (fertilizin)

FIGURES 11–13. Spermatozoa of *S. pulcherrimus;* freshly suspended sperm fixed on collodion membrane, showing various degrees of acrosome reaction.

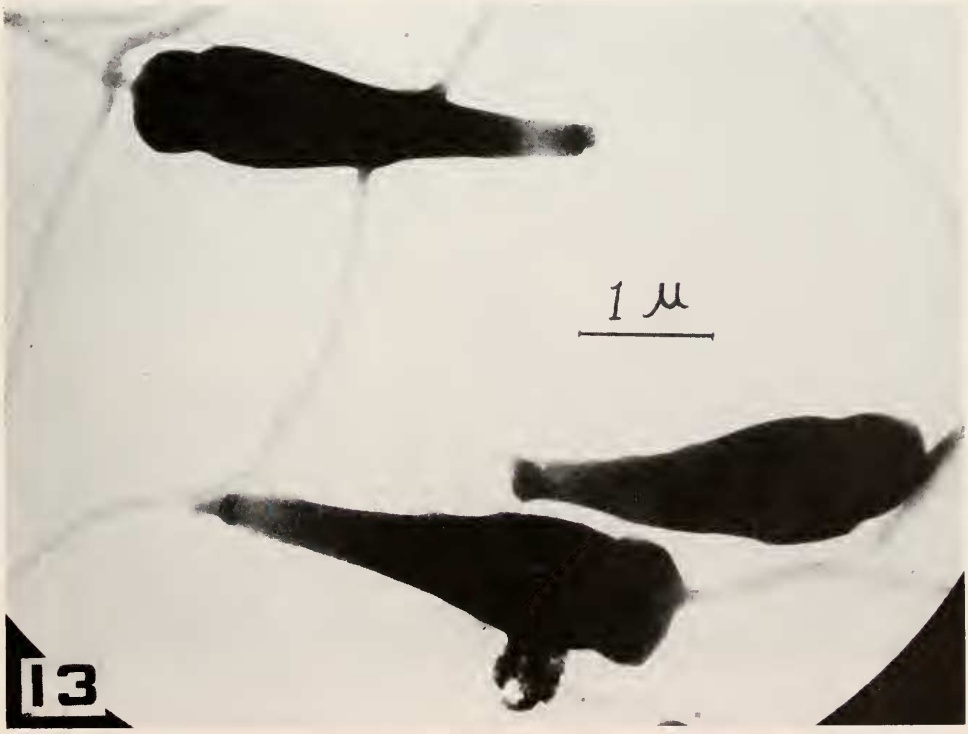
FIGURE 11. Acrosome partially reacting to stimulus of its own tail.

FIGURE 12. Acrosome of sperm at right completely reacted to contact with collodion membrane.

FIGURE 13. These three spermatozoa were rotating about their tips on the collodion membrane when fixed. (This photograph was intentionally under-exposed to show differences in density of acrosome region. Fully exposed print shows acrosome substance spread on collodion membrane around points of attachment, similar to condition in Figure 15.)


1 μ
11


1 μ
12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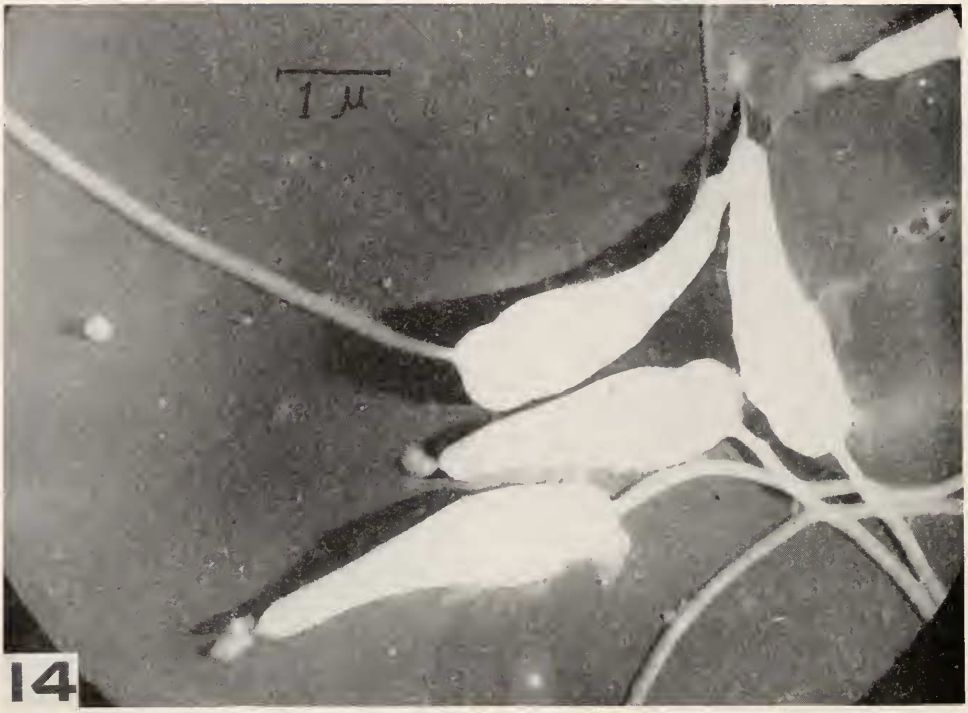

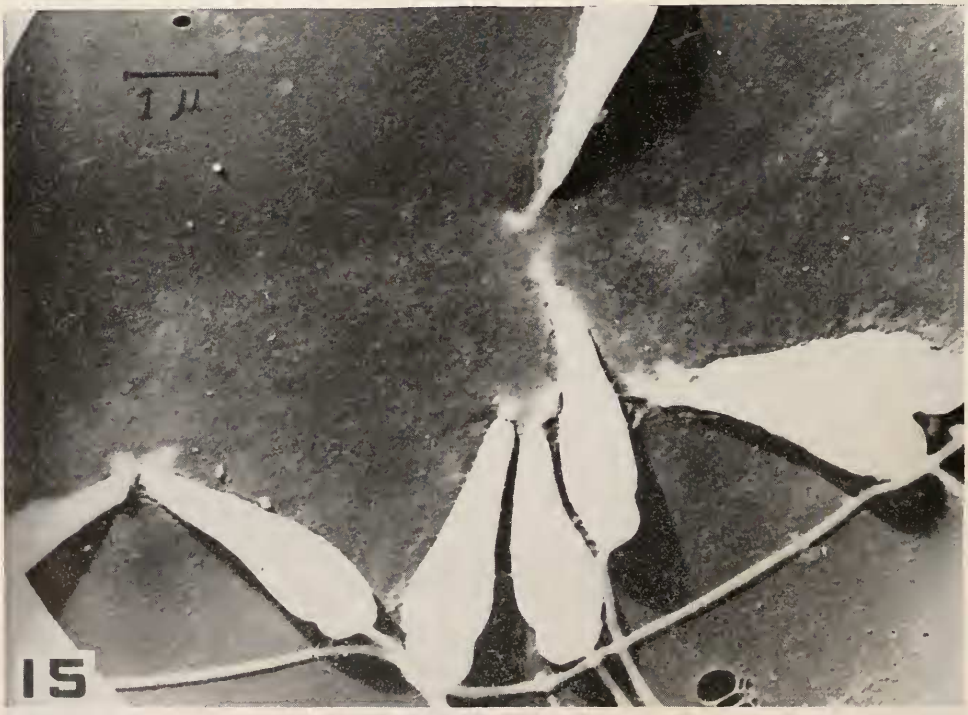

FIGURES 14 and 15. Spermatozoa fixed while rotating around tips on collodion membrane. Acrosome substance newly exposed (Fig. 14) and in several degrees of spreading on collodion surface (Fig. 15). Shadowing with Cr_2O_3; $\tan^{-1} = \frac{1}{3}$.

would seem to furnish an adequate explanation for the subsequent failure of the spermatozoan to effect penetration of the egg.

Krauss (1950a) has worked out in great detail the conditions under which egg-membrane lysin is obtainable from spermatozoa of the keyhole limpet. One of his most striking cases, that of increased pH, coincides with the result reported in this paper, and there is nothing in his other observations which is contrary to the suggestions made here. Coordination of the experimental conditions might very well bring out an interesting positive agreement between the two sets of results.

The writer wishes to express sincerest gratitude to Prof. A. Takamiya, of Tokyo Institute of Technology, for his enthusiastic encouragement and kindness in arranging for the use of the Institute's electron microscope; and to Mr. H. Akabori, of the Institute, who has cooperated most generously in preparing and photographing the material. The writer's thanks are also due in large measure to the Director and Staff of the Misaki Marine Biological Station for their unfailing courtesy in sharing the Station's facilities; and finally to Drs. C. B. Metz, D. Mazia and K. Dan, for their exceedingly helpful criticism of the manuscript.

Summary

1. Spermatozoa of two sea urchin species were fixed with formalin in sea water, egg-water and sea water of pH 9.2, and observations of fixed specimens made with phase contrast and electron microscopes were compared with phase contrast observation in the living state.

2. In response to egg-water, a reaction is called forth which is interpreted as a breakdown of the acrosomal membrane, by which a labile mass of substance is exposed at the tip of the spermatozoan head.

3. This same reaction is induced by exposure to sea water of pH 9.2, and by contact with solid surfaces.

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